

DISSOCIATION CONSTANTS OF THE COMPONENTS IN THE ENZYMATIC SYNTHESIS OF ASPARTAME PRECURSOR

MAKOTO HIRATA*, NOZOMU SHIBUYA AND AKIRA HIRATA

Department of Chemical Engineering, Waseda University, Tokyo 169

MASAHICO INAMO

Department of Chemistry, Faculty of Education, Aichi University of Education, Aichi 448

NORIYUKI NAKASUKA

Faculty of General Education, Gifu University, Gifu 501-11

Key Words: Biochemical Engineering, Aspartame Precursor, Peptide Synthesis, Dissociation Constant, Ionization Constant, Equilibrium Constant, Thermolysin

Introduction

In recent years, since peptide production by the enzymatic method was developed into a commercial process, there has been great interest in the enzymatic synthesis of peptide. The precursor of the synthetic sweetener aspartame, *N*-(benzyloxycarbonyl)-L-aspartyl-L-phenylalanine methyl ester (abbreviated as Z-APM), is one of the novel peptides which have been studied for synthesis by the enzymatic method. Z-APM can be synthesized enzymatically from *N*-(benzyloxycarbonyl)-L-aspartic acid (abbreviated as Z-L-Asp) and L-phenylalanine methyl ester (abbreviated as L-Phe-OMe) by thermolysin²⁻⁶⁾. In this reaction, since the substrates and the product partially dissociate into ions, the values of the acidic dissociation constant, K_a , of the components are important for reaction kinetics and equilibrium. Nakanishi, Matsuno *et al.*⁵⁾ obtained the values of K_a in the aqueous/organic biphasic system and analyzed the kinetics and equilibrium of the synthesis in the system. The precise values in the aqueous system, however, have not yet been measured for use in the analysis of the reaction kinetics and equilibrium. In this work the K_a values were measured by potentiometric titration, and the reaction equilibrium of enzymatic synthesis of aspartame precursor was analyzed in aqueous system with these values.

1. Experimental

1.1 Measurement of dissociation constants

Measurement of K_a values was carried out at temperatures of 25.0°C and 35.0°C, controlled within $\pm 0.1^\circ\text{C}$. The ionic strength, I , was adjusted to 0.100 mol/l with sodium perchlorate (recrystallized twice). The hydrogen ion concentration was measured by a Metrohm pH meter Model E654 with a combination glass electrode. An aqueous solution of perchloric acid

* Received October 8, 1993. Correspondence concerning this article should be addressed to M. Hirata, Postdoctoral Fellow of the Japan Society for the Promotion of Science.

(10.00×10^{-3} mol/l, $I = 0.100$ mol/l) was used as the pH standard solution. The glass electrode was calibrated by testing the response of the electromotive force value to the hydrogen ion concentration, where the pH standard solution was titrated with aqueous sodium hydroxide (carbonate-free) at a respective temperature. During the potentiometric titration, the concentrations of the components were as follows: Z-APM, *ca.* 0.3×10^{-3} mol/l; Z-L-Asp, *ca.* 3×10^{-3} mol/l; L-Phe-OMe, *ca.* 6×10^{-3} mol/l.

1.2 Measurement of equilibrium constants

To obtain the values of apparent equilibrium constant, K_{eq}^{app} , under various pH conditions, reactions were carried out at 40°C in 0.1 mol/l Mes-NaOH buffer (in the presence of 0.01 mol/l CaCl_2). The initial concentrations were as follows: Z-APM, *ca.* 1×10^{-3} mol/l; Z-L-Asp, *ca.* 20×10^{-3} mol/l; L-Phe-OMe, *ca.* 20×10^{-3} mol/l; Thermolysin, *ca.* 2.7×10^{-3} mol/l. The equilibrium concentrations of the substrates and the product were measured by HPLC performed with Tosoh UV-8000 detectors and CCPD solvent delivery systems (1.0 ml/min). For the substrates, TSKgel G2000SW (Tosoh: 7.5 mm i.d. \times 300 mm) and acetonitrile/0.3 mol/l CH_3COONa (7:3, v/v; pH5.68) were used, and the absorbance was detected at 260 nm. For the product, TSKgel ODS-80TM (Tosoh: 6.0 mm i.d. \times 150 mm) and acetonitrile/0.12 mol/l CH_3COONa (5:5, v/v; pH4.8) were used, and the absorbance was detected at 258 nm.

2. Results and Discussion

2.1 Dissociation constants

The values of K_a obtained are shown in Table 1, and the calculated values of the thermodynamic parameters are shown in Table 2. K_a values at 40°C were calculated from the thermodynamic parameters and are shown in Table 2.

The calculated values of $\text{p}K_a$ in aqueous solution as shown in Table 2 differ slightly from the values of $\text{p}K_a$

Table 1. Obtained values of dissociation constants (I = 0.100 mol/l with NaClO₄)

Component	T [°C]	K _a [mol/l]	pK _a [-]
Z-APM	25.0	(9.23 ± 0.14) × 10 ⁻⁵	4.035 ± 0.007
	35.0	(9.01 ± 0.14) × 10 ⁻⁵	4.045 ± 0.007
Z-L-Asp*	25.0	(6.79 ± 0.05) × 10 ⁻⁴	3.168 ± 0.003
		(2.45 ± 0.01) × 10 ⁻⁵	4.611 ± 0.002
	35.0	(6.87 ± 0.02) × 10 ⁻⁴	3.163 ± 0.001
		(2.44 ± 0.01) × 10 ⁻⁵	4.613 ± 0.002
L-Phe-OMe	25.0	(8.33 ± 0.01) × 10 ⁻⁸	7.079 ± 0.001
	35.0	(1.61 ± 0.01) × 10 ⁻⁷	6.793 ± 0.003

* Z-L-Asp is dissociated into Z-L-Asp⁻ and Z-L-Asp²⁻ by two steps

Table 2. Obtained values of thermodynamic parameters and calculated values of dissociation constants at 40°C (I = 0.100 M with NaClO₄)

Component	ΔH° [kJmol ⁻¹]	ΔS° [JK ⁻¹ mol ⁻¹]	K _a (40°C)	pK _a (40°C)
Z-APM	-1.84	-83.4	8.93 × 10 ⁻⁵	4.05
Z-L-Asp	0.89	-57.7	6.88 × 10 ⁻⁴	3.16
	-0.31	-89.3	2.44 × 10 ⁻⁵	4.61
L-Phe-OMe	50.3	33.3	2.20 × 10 ⁻⁷	6.66

obtained by the solvent extraction method^{2,5)}, since the previous values included the effect of saturation of the organic solvents.

2.2 Equilibrium constants

The equilibrium constant in the enzymatic synthesis of aspartame precursor was studied in terms of the K_a values obtained.

The apparent equilibrium constant, K_{eq}^{app}, is based on the total concentrations obtained directly by HPLC, and is defined as follows:

$$K_{eq}^{app} = \frac{[Z-APM]_t [H_2O]}{[Z-Asp]_t [PM]_t} \quad (1)$$

In this reaction, since both ionized and nonionized forms of the substrates and the product exist under the conditions of pH 4~7, four couples of the equilibrium should be taken into consideration. In the case using the equilibrium constant for the equilibrium based on the nonionized components, K_{eq}, Eq. (1) can be represented as

$$K_{eq}^{app} = K_{eq} \cdot \frac{1 + K_a^A / [H^+]}{(1 + [H^+] / K_a^B) \{1 + (1 + K_{a2}^C / [H^+]) \cdot K_{a1}^C / [H^+]\}} \quad (2)$$

where the superscripts have the following meanings:

A: Z-APM, B: L-Phe-OMe, C: Z-L-Asp.

The relation between the apparent equilibrium constants, K_{eq}^{app} and pH, is shown in Fig. 1. The value of K_{eq} was obtained as 97.7 × 10³ by the least-squares method with the values of K_a shown in Table 2 and [H₂O] = 55.1 M at 40°C. Nakanishi, Matsuno *et al.*⁵⁾ have reported the equilibrium constant for nonionized forms (K_{eq} in this paper) as 102 × 10³ in aqueous solution saturated with ethyl acetate. A similar value of K_{eq} was obtained in this study. In Fig. 1, the solid line means the results calcu-

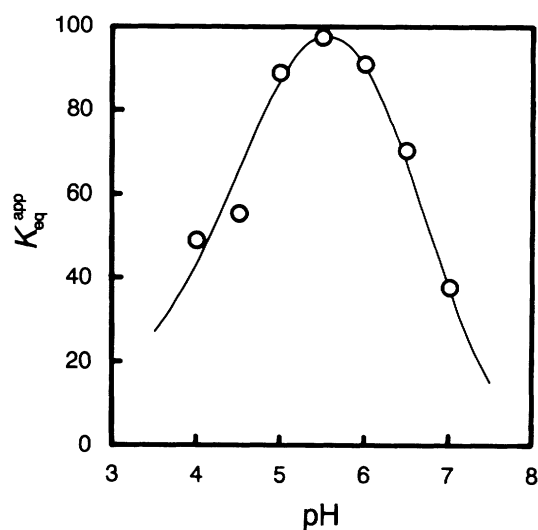


Fig. 1 Effects of pH on apparent equilibrium constant, K_{eq}^{app}, at 40°C in the thermolysin-catalyzed enzymatic synthesis of aspartame precursor

lated by Eq. (2), and the key means the results calculated by Eq. (1) with measured values of total concentration. The calculated results agree well with the experimental ones.

Conclusion

The dissociation constants of the components in the enzymatic synthesis of aspartame precursor were measured at 25°C and 35°C, and thermodynamic parameters were obtained from these values. The apparent equilibrium constants of the thermolysin-catalyzed enzymatic synthesis of aspartame precursor at 40°C were calculated with the dissociation constants obtained from the thermodynamic parameters, and the results agreed well with the experimental ones.

Nomenclature

K _{eq}	= equilibrium constant for nonionized component	[-]
K _{eq} ^{app}	= apparent equilibrium constant	[-]
K _a ⁱ , K _{a1} ⁱ , K _{a2} ⁱ	= acidic dissociation constant	[mol/l]
ΔH°	= enthalpy change at atmospheric pressure	[kJmol ⁻¹]
ΔS°	= entropy change at atmospheric pressure	[JK ⁻¹ mol ⁻¹]

<Subscript>

t = total

<Superscript>

i = component (A: Z-APM, B: L-Phe-OMe, C: Z-L-Asp)

Literature Cited

- 1) Carpenter, F.H.: *J. Am. Chem. Soc.*, **82**, 1111-1122 (1960)
- 2) Hirata, A. and M. Hirata, M.: *Kagaku Kogaku Ronbunshu*, **17**, 511-517 (1991)
- 3) Hirata, A., M. Hirata, H. Furuzawa and N. Honda: *Kagaku Kogaku Ronbunshu*, **17**, 586-588 (1991)
- 4) Hirata, A., M. Hirata and N. Honda: *Proc. 4th World Congress Chem. Eng.*, **2**, 7.2-44 (1991)
- 5) Nakanishi, K., Y. Kimura and R. Matsuno: *Eur. J. Biochem.*, **161**, 541-549 (1986)
- 6) Oyama, K. and K. Kihara: *Kagaku Sousetsu*, **35**, 195-203 (1982)